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TOBACCO INDUSTRY RESEARCH COMMITTEE

150 East Forty Second Street

New York 17, N.Y.

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Renewal
Application for Research Grant

Date: December 8, 1959

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2. Title: Associate Professor of Pharmacology

3. Institution & Address: Department of Pharmacology and Experimental Therapeutics
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4. Project or Subject: Pharmacological analysis of the mechanism by which nicotine impairs transmission through autonomic ganglia.

5. Detailed Plan of Procedure: In previous reports of the present project, we described the evidence which lead us to believe that nicotine prevents transmission of nerve impulses through autonomic ganglia by a mechanism hitherto never ascribed to nicotine. In brief, we believe that nicotine can modify cardiovascular and other autonomic effector activity by preventing release of acetylcholine from nerve endings in autonomic ganglia, and thereby prevent nerve impulse transmission. We have not attempted to investigate the mechanism by which nicotine causes stimulation of autonomic ganglia.



The data upon which this hypothesis is based were obtained by recording the mechanical responses of the cat nictitating membrane to high and low frequencies of preganglionic nerve stimulation. These data were compared with mathematical formulations of effects to be expected were a hypothetical drug to impair ganglionic transmission by impairing acetylcholine release or, alternatively, by preventing access of acetylcholine to postsynaptic cell receptors. The excellent fit of the data obtained with nicotine, tetraethylammonium ions, morphine, and hexamethonium ions to the theoretical formulations led to our establishing the hypothesis summarized above.

During the past year the over-all object of our work has been to obtain direct experimental evidence that impaired ganglionic transmission produced by nicotine, tetraethylammonium or morphine was accompanied by a decrease in the rate of release of acetylcholine from the ganglion, and that hexamethonium blocked ganglionic transmission without affecting acetylcholine release. The work was performed in two parts:

(1) A method was developed, using the isolated rat ileal strip, which permitted the bioassay of quantities of acetylcholine as low as one one-thousandth of a microgram. The assays could be performed without interference by the presence of nicotine, tetraethylammonium, or hexamethonium in quantities one thousand to one hundred thousand times as large. Using this technique it was possible to demonstrate that nicotine in concentrations of 10 mcg/ml completely suppressed the release of acetylcholine from isolated guinea pig ileal segments; no such suppression of acetylcholine release was obtained with concentrations of tetraethylammonium or hexamethonium as large as 1 mg/ml. A summary of this work was submitted to the Scientific Advisory Board on May 7, 1959.

(2) The superior cervical ganglion of the anesthetized cat was isolated and perfused with Ringer's solution using, essentially, the method of Kibjakov. Using this technique, the ganglion and its nerve supply, and the nerve supply to the nictitating membrane remain functional for reasonably long periods of time. The effluent from the ganglion was collected before, during, and after various procedures, including the injection of nicotine, and the acetylcholine content of each sample was assayed using the bioassay technique described above. A brief summary of the experimental results is appended to this application; a detailed summary will be submitted upon the expiration of the current research grant.

We propose to continue our research by extending our investigations of the effects of nicotine and other ganglionic "blocking" agents on the output of acetylcholine from the isolated, perfused superior cervical ganglion of the cat. Specifically, at least four kinds of experiments need to be performed:

1. Additional experiments must be performed to confirm the observed effects of nicotine in decreasing the output of acetylcholine from the ganglion. At the present time, satisfactory experiments with nicotine have been performed in three cats; for only two experiments could the results of the bioassay be interpreted unequivocally. For both experiments, the data indicate that acetylcholine output from the ganglion was decreased abruptly and remarkably at the time ganglionic transmission was prevented by the injected nicotine.

2. Experiments must be performed with agents such as morphine and tetraethylammonium, which we anticipate will prevent ganglionic transmission and decrease acetylcholine output as nicotine seems to do. Experiments must be performed using hexamethonium to produce ganglionic blockade; we anticipate such a blockade to be unaccompanied by significant changes in acetylcholine release. In one experiment performed so far with hexamethonium, it was found that the acetylcholine content of the effluent from the ganglion was too low, even before drug administration, to permit reliable assays.

   It is obviously crucial to our hypothesis concerning the mechanism of action of nicotine in preventing ganglionic transmission that we demonstrate that agents which affect the response of the nictitating membrane to high and low frequency stimulation, as nicotine does, also have effects on acetylcholine output like those of nicotine. Similarly, a counter proof is required; hexamethonium and nicotine affect the response of the membrane differently, and we must demonstrate different effects of the two on acetylcholine output.

3. Experiments must be performed to determine the significance of changes in vascularity in the perfused tissues in altering the quantity of acetylcholine in the effluent. In contrast to our expectation based on a study of the literature, we have found it impossible, thus far, to isolate the afferent and efferent vascular supply of the ganglion from the systemic circulation, or from the circulation to other tissues which may act as potential sources at least of acetylcholine. Furthermore, we have observed that injection of nicotine to the ganglion causes changes in resistance to perfusion and changes in the fraction of infused fluid recovered as effluent; these results may indicate that acetylcholine detected in "control" samples of effluent may be derived in whole or in part from areas of the cat quite different from those which supply the acetylcholine which is detected in effluent collected during the course of the nicotine effect.

The effect of experimental manipulations in changing the areas of perfusion and drainage in the Kibjakov preparation and the consequent effects of such changes on the apparent output of "transmitter" from the "ganglion" seems not to have been discussed in previous publications. This is most remarkable since the theory of acetylcholine as the neurohumoral transmitter in ganglia rests in part on the inference that changes in acetylcholine output from the "ganglion" are related only to the functional state of pre-ganglionic nerve terminals in the ganglion alone.

We hope to investigate the relationship between the vascularity of the perfused area and acetylcholine output in experiments in which:

a. We study the effects of surgical extirpation of the ganglion or pre- and post-ganglionic nerve section on acetylcholine output and resistance to perfusion.

b. Vasoconstriction in the perfused area is produced by a substance such as vasopressin which has no known effects on acetylcholine release.

c. An attempt is made to influence acetylcholine output with agents such as morphine which may well be given without producing significant effects on the vasculature. Any effects of morphine on acetylcholine output may reasonably be expected to outlast effects on vessels such as might be produced by histamine release.

4. Experiments should be performed to identify the spasmogen assayed in our rat ileal preparation with acetylcholine. Like the activity of acetylcholine, the activity of the spasmogen we assay as acetylcholine is destroyed by boiling of the effluent or prevented by atropinization of the ileum. However, in our 4-point-assay design, dose-effect curves for the spasmogen have slopes consistently higher than the slopes of the curves for the standard, acetylcholine. According to the logic of bioassay, this indicates that the spasmogenic activity of the samples does not reside in a molecular species identical with acetylcholine. Previous investigators have routinely used 3-point-assays of the effluent to

detect "acetylcholine." Such a technique permits the use of small effluent volumes and permits a larger proportion of "successful" assays and experiments, but precludes identification of the standard with the unknown agent.

Resolution of the problem of the non-parallelism of the dose-effect curves of the standards and unknown agents may take two courses:

a. Alteration of the experimental design--by using other anesthetic agents, anti-coagulant drugs, or anti-cholinesterase drugs in the perfusing fluid--to determine whether the lack of parallelism is adventitious.

b. Concentration of the effluent and an attempt to identify acetylcholine in it by chemical or enzymatic means.

The methods presently available to us seem adequate to determining whether administration of nicotine, or other agents which impair ganglionic transmission, can alter both ganglionic transmission and "acetylcholine" output in the effluent. An adequate biological interpretation of these facts, an attempt to determine causal relations between them, requires further identification of the ganglion as the only significant source of the spasmogen assayed and more certain identification of the spasmogen with acetylcholine.

6. Budget Plan:

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| Salaries | $ 4,500. * |
| Expendable Supplies | 2,000. |
| Permanent Equipment | 2,000. |
| Overhead | 1,365. |
| Other | 600. |
| Total | $ 10,465. |

* E. W. Pelikan $1,000.
Research Asst. 3,500.

7. Anticipated Duration of Work:

Two years; the budget plan above applies only to the first year of the research.

8. Facilities and Staff Available:

Facilities and staff of the Department of Pharmacology and Experimental Therapeutics of the Boston University School of Medicine. The staff members are competent and well equipped to aid the course of this study, especially insofar as special knowledge of biochemical and physical chemical methods might be required, and insofar as special techniques might be required to record and study cardiovascular phenomena. No salaries are requested for persons who might aid the work in this way.

9. Additional Requirements:

None.

10. Additional Information (Including relation of work to other projects and other sources of supply):

This proposed research program is the direct outgrowth and continuation of research now being conducted by the applicant under a grant from the Tobacco Industry Research Committee. The work is also an extension of previous investigation by the applicant; these investigations have included studies of the pharmacology of the neuromuscular junction in several species, studies of the structure-activity relationships among neuromuscular blocking agents, and studies of the actions of nicotine and sympathomimetic amines on isolated intestinal strips, the frog rectus abdominis muscle, and on the superior cervical ganglion of the cat.

Application for support of this work has not been made to any other agencies.

The applicant will spend about three-fourths of his time on these investigations.

/s/ Edward W. Pelikan

/s/ K. M. Haetzfeld